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TECHNICAL MANUSCRIPT 142

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DEFINED MEDIUM FOR MAINTAINING CHICK FIBROBLAST MONOLAYERS

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ABSTRACT

A defined maintenance medium that supports monolayers of chick fibroblast (CF) cells in a viable state and permits the formation of plaques by Venezuelan (VEE) and eastern (EEE) equine encephalitis viruses is described. The requirements for the individual constituents of a defined medium for maintaining CF monolayer viability and for plaque formation were determined by systematically omitting one component at a time from an otherwise complete medium. In the absence of amino acids, the monolayers died. Histidine monohydrochloride combined with L-cystine or L-cysteine were found to be the only amino acids necessary to maintain viable monolayers and permit plaque formation by VEE and EEE viruses. Any other amino acids, alone or in combination, failed to maintain the CF cell cultures. Glucose, the sole carbohydrate source, and sodium chloride were also necessary for cell survival.

I. INTRODUCTION

The composition of a medium capable of supporting viable monolayers of chick fibroblast (CF) cells and of permitting the formation of plaques by Venezuelan (VEE) and eastern (EEE) equine encephalitis viruses has recently been investigated. The investigations were conducted by systematically omitting one component at a time from an otherwise complete, defined medium.* Histidine monohydrochloride combined with L-cystine or L-cysteine were found to be the only amino acids necessary to maintain viable monolayers and permit plaque formation by VEE and EEE. Glucose, a carbohydrate source, and sodium chloride were also necessary in the medium to maintain the CF cultures. The inorganic salts KCl, $MgCl_2$, and $CaCl_2$ were not necessary for cell survival, but the addition of these salts improved the vital staining properties of the cells.

On the basis of these investigations the medium presented in Table I was derived. In this medium, normal cells remained viable for as long as ten days, and plaque counts with VEE or EEE virus equaled those obtained in a more complex medium consisting of lactalbumin hydrolyzate, yeast extract, and Hanks' balanced salt solution.

TABLE I. COMPOSITION OF MINIMAL DEFINED MAINTENANCE MEDIUM

Material	Milligrams Per Liter
Sodium chloride	7400
L-cystine	75
L-histidine·HCl	60
Glucose	1000
NaH_2PO_4	100
$CaCl_2$	265
$MgCl_2$	275
KCl	400
Sodium bicarbonate	1400

* Nagle, S.C.; Tribble, H.R., Jr.; Anderson, R.E.; and Gary, N.O. "A chemically defined medium for growth of animal cells in suspension," Proc. Soc. Expt. Biol. Med. 112:340-344, 1963.

II. MATERIALS AND METHODS

To prepare monolayers, 20 to 30 million cells from minced, trypsinized, ten-day chick embryos were added to 60-mm Petri plates. Confluent cell sheets were obtained within 24 hours in a growth medium consisting of 0.5 per cent lactalbumin hydrolyzate, 0.1 per cent yeast extract, Hanks' balanced salt solution, and 10 per cent calf serum. The monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.4, and then infected with appropriate dilutions of VEE or EEE virus prepared in beef heart infusion broth (BHIB), unless otherwise stated. All media to be tested were prepared as overlays in 1.1 per cent agar. Plates were incubated at 37°C in an atmosphere of 5 per cent CO_2 and 95 per cent air for two days. The monolayers were then stained with 1:10,000 neutral red in 1.1 per cent agar to determine cell viability and to detect plaques.

III. RESULTS

The medium described by Nagle and co-workers for growing suspension cell cultures was modified by increasing the sodium bicarbonate concentration and by including seven additional amino acids. The complete medium, presented in Table II, includes a total of 19 amino acids, glutamine, a glucose-salts mixture, sodium bicarbonate, a vitamin mixture, antibiotics, and dye. This complete medium, when used in an agar overlay for CF monolayers infected with VEE or EEE virus, maintained the cells in a viable condition and permitted maximum plaque formation. The requirement for the different constituents of this medium for cell viability and plaque formation was systematically examined by omitting one component at a time from an otherwise complete growth medium.

Deletion of all the amino acids from the complete medium caused the death of uninfected cell monolayers within 48 hours and no plaques were detectable in cultures that had been infected with VEE or EEE virus. If all the amino acids were retained in the medium, the monolayers remained viable and supported plaque formation by VEE and EEE viruses.

The individual amino acid requirements for monolayer maintenance and plaque formation are shown in Table III. Various agar overlay media were prepared by omitting single amino acids from the complete medium. The results show that the CF monolayers died only in the absence of cysteine or cystine. None of the other amino acids was necessary for plaque formation or maintenance of cell viability. Cystine, being more stable in stock solution than cysteine, was selected to be used routinely in all subsequent media described in this report.

TABLE II. NAGLE'S CHEMICALLY DEFINED MEDIUM MODIFIED FOR AGAR OVERLAY

Amino Acids	Milligrams per Liter	Glucose-salts Mixture	Milligrams per Liter
L-arginine·HCl	100	NaCl	7400
L-cysteine·HCl	75	KCl	400
L-histidine·HCl	60	$NaH_2PO_4 \cdot H_2O$	100
L-isoleucine	150	$CaCl_2 \cdot 2H_2O$	265
L-leucine	300	$MgCl_2$	275
L-lysine·HCl	300	Glucose	1000
L-methionine	60	Sodium pyruvate	110
L-phenylalanine	120		
L-threonine	135	Vitamins	
L-tryptophane	60		
L-tyrosine	120	D-biotin	1.0
L-valine	150	Choline Cl	1.0
L-alanine*	120	Niacinamide	1.0
L-aspartic acid*	270	Ca pantothenate	2.0
L-cystine*	75	Pyridoxine·HCl	1.0
L-glutamic acid*	315	Thiamine·HCl	1.0
Glycine*	60	i-inositol	1.0
L-proline*	115	Riboflavin	0.1
L-serine*	150	B_{12}	0.002
		Folic Acid	1.0
L-glutamine	450		
		Antibiotics, etc.	
Bicarbonate buffer		Streptomycin	100
		Phenol red	10
$NaHCO_3$*	1400	Agar	11,000
		Penicillin	100,000 units/liter

* Modifications or additions to Nagle's medium.

TABLE III. EFFECT OF OMISSIONS OF INDIVIDUAL AMINO ACIDS FROM DEFINED MEDIUM UPON PLAQUE FORMATION

Essential Amino Acid Omitted	Monolayer Viability	VEE Plaques	EEE Plaques
L-cysteine·HCl	-a/	-	-
L-cystine	-	-	-
L-arginine·HCl	+	+	+
L-histidine·HCl	+	+	+
L-isoleucine	+	+	+
L-leucine	+	+	+
L-lysine	+	+	+
L-methionine	+	+	+
L-phenylalanine	+	+	+
L-threonine	+	+	+
L-tryptophane	+	+	+
L-tyrosine	+	+	+
L-valine	+	+	+
L-glutamine	+	+	+
Glycine	+	+	+
L-alanine	+	+	+
L-serine	+	+	+
L-aspartic	+	+	+
L-glutamic	+	+	+
L-proline	+	+	+
Complete	+	+	+

a. + Monolayers viable or plaques present.
- Monolayers dead or no plaques counted.

The requirement of CF monolayers for other individual components of the medium was also determined. With cystine as the only amino acid present, the other components were deleted one at a time to determine the effect on cell viability and plaque formation. The results are shown in Table IV. In the absence of sodium chloride or glucose the monolayers died. Omission of the vitamin mixture, salts, and sodium pyruvate had no adverse effect upon the monolayer viability or the plaque count. However, in the absence of potassium chloride, magnesium chloride, or calcium chloride, it appeared that the monolayers stained poorly with neutral red and observation of plaques was more difficult.

TABLE IV. THE REQUIREMENTS OF CHICK FIBROBLAST MONOLAYERS FOR THE INDIVIDUAL COMPONENTS OF THE DEFINED MEDIUM

Medium[a]	Monolayer Viability	VEE Plaques	EEE Plaques
Complete	+[b]	+	+
Complete less NaCl	-	-	-
Complete less glucose	-	-	-
Complete less vitamins	+	+	+
Complete less KCl	+	+	+
Complete less NaH_2PO_4	+	+	+
Complete less sodium pyruvate	+	+	+
Complete less $MgCl_2$	+	+	+
Complete less $CaCl_2$	+	+	+

a. Cystine was the sole amino acid added to all media.
b. + Monolayers viable or plaques present.
- Monolayers dead or no plaques observed.

The type of diluent used in preparing the virus dilutions had a profound effect upon the ability of a cystine basal medium (glucose, sodium chloride, a salts mixture, and cystine) to maintain the viability of CF cell monolayers. When virus dilutions were prepared in BHIB and overlayed with the cystine basal medium, the monolayers remained viable and supported plaque formation by either VEE or EEE virus. However, when virus dilutions were made in gel saline or PBS the cell monolayers died. Moreover, if cystine was omitted from the basal medium and dilutions of virus that were used as inocula were prepared in BHIB, the monolayers died. These data indicated that the BHIB used for virus dilution in these studies was deficient in cystine and cysteine and that additional nutrients present in BHIB were necessary for monolayer survival.

If dilutions of virus were prepared in gel saline for use as inocula, cell monolayers survived and plaques formed provided that cystine and one of several other amino acids were present. These results are shown in Table V.

TABLE V. AMINO ACID REQUIREMENTS FOR CHICK FIBROBLAST CELL MONOLAYERS WHEN VIRUS INOCULA WERE PREPARED IN GEL SALINE

Amino Acid Additions to Cystine Basal Medium	Number of Plaques Per Plate	
	VEE	EEE
None	-[a]	-
L-histidine·HCl	62	139
L-methionine	26	55
L-threonine	15	45
L-tryptophane	-	-
L-arginine	-	-
L-cysteine	-	-
L-isoleucine	-	-
L-leucine	-	-
L-lysine	-	-
L-phenylalanine	-	-
L-tyrosine	-	-
L-valine	-	-
Control[b]	51	123

a. Monolayer dead.
b. Control medium consisted of 0.5% lactalbumin hydrolyzate
0.1% gelatin
0.1% yeast extract
Hanks' balanced salt solution
1.1% agar

If cystine was the only amino acid present, the CF monolayers died, as stated before. However, when histidine was added to the cystine basal medium the monolayers remained viable and supported plaque formation. The plaque count on the histidine-cystine medium equaled that of the control medium. Methionine and threonine in combination with cystine supported viable monolayers, but the number of VEE or EEE plaques produced was reduced. These data demonstrate that two amino acids, cystine and histidine, are essential for maintaining viable CF cell monolayers.

The composition of the defined maintenance medium has been shown in Table I. Cystine and histidine were the only amino acids added. Sodium acid phosphate, although not necessary for monolayer maintenance, was added to enhance the buffering capacity of this medium. Magnesium chloride, calcium chloride, and potassium chloride were added because their presence improved contrast and made plaques much easier to detect.

Plaque formation by VEE and EEE viruses was not adversely affected by this medium. Table VI presents the plaque counts obtained on the defined maintenance medium and compares them with those obtained with a complex medium containing 0.5 per cent lactalbumin hydrolyzate, 0.1 per cent gelatin, 0.1 per cent yeast extract, and Hanks' balanced salt solution. The values shown here are the average plaque counts of three or four replicate plates. The data show some variation, but the average ratio of counts on complex medium to those on defined maintenance medium approximated 1.0, indicating that plaque counts on both media were equivalent.

TABLE VI. COMPARISON OF PLAQUE COUNTS OF VEE AND EEE VIRUSES ON CHICK EMBRYO CELL MONOLAYER OVERLAYED WITH LACTALBUMIN HYDROLYZATE MEDIUM AND WITH MINIMAL MAINTENANCE MEDIUM

Run	VEE			EEE		
	Lactalbumin Hydrolyzate	Minimal	Ratio[a]	Lactalbumin Hydrolyzate	Minimal	Ratio[a]
1	32	25	0.78	36	33	0.92
2	110	134	1.21	102	89	0.87
3	105	73	0.70	36	43	1.19
4	NT[b]	NT	-	124	111	0.89
5	171	210	1.33	153	175	1.14
6	36	63	1.75	141	137	0.97
7	75	69	0.92	102	87	0.78
8	127	108	0.85	218	203	0.93
Average			1.08			0.96

a. Ratio = $\frac{\text{Plaque count on minimal defined maintenance medium}}{\text{Plaque count on lactalbumin hydrolyzate medium}}$

b. Not tested.

IV. SUMMARY

A defined medium for the maintenance of CF cell monolayers was described. The components of the medium, essential with respect to cell viability and plaque formation, consisted only of cystine, histidine, glucose, and sodium chloride. This medium maintained CF monolayers in a viable state and permitted the formation of plaques by VEE and EEE viruses. The number of virus plaques formed when this medium was used in the agar overlay equaled that formed with a richer, more complex medium. This maintenance medium was developed as a tool to determine the nutritional factors that influence viral plaque formation. Various modifications of this medium are now being tested to determine their effect upon cell viability and plaque count of VEE and EEE viruses.